**RIP1/3-dependent programmed necrosis induces intestinal injury in septic rats**

<table>
<thead>
<tr>
<th>Journal:</th>
<th><em>Acta Biochimica et Biophysica Sinica</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Manuscript ID</td>
<td>ABBS-2023-275.R4</td>
</tr>
<tr>
<td>Manuscript Type:</td>
<td>Original Article</td>
</tr>
<tr>
<td>Date Submitted by the Author:</td>
<td>10-Oct-2023</td>
</tr>
<tr>
<td>Complete List of Authors:</td>
<td>Ye, Siting; Fuzhou Second General Hospital of Xiamen University, School of Medicine, Xiamen University, Fuzhou Second General Hospital Liu, Yuxiao; Fuzhou Second General Hospital of Xiamen University, School of Medicine, Xiamen University, Fuzhou Second General Hospital Qi, Xinxin; Fuzhou Second General Hospital of Xiamen University, School of Medicine, Xiamen University, Fuzhou Second General Hospital Zhuang, Liangming; Fuzhou Second General Hospital of Xiamen University, School of Medicine, Xiamen University, Fuzhou Second General Hospital Gu, Zhongmin; Fuzhou Second General Hospital of Xiamen University, School of Medicine, Xiamen University, Fuzhou Second General Hospital</td>
</tr>
<tr>
<td>Keywords:</td>
<td>RIP1, RIP3, MLKL, ROS, programmed cell necrosis, sepsis</td>
</tr>
</tbody>
</table>
Original Article

RIP1/3-dependent programmed necrosis induces intestinal injury in septic rats

Siting Ye, Yuxiao Liu, Xinxin Qi, Liangming Zhuang, and Zhongmin Gu*

Department of Intensive Care Unit, Fuzhou Second General Hospital of Xiamen University, School of Medicine, Xiamen University, Fuzhou Second General Hospital, Fuzhou 350007, China.

*Correspondence address. Tel: +86-591-22169268; E-mail: Guzhongmin2023@163.com

Received: 06-Jun-2023

Accepted: 25-Aug-2023

Running title: RIP1/3-dependent programmed necrosis induces intestinal injury

The Author(s) 2023. This is an open access article distributed under the terms of the Creative Commons Attribution-NonCommercial-NoDerivs License (https://creativecommons.org/licenses/by-nc-nd/4.0/).

Abstract

The regulation of various types of cell death may help to restore the normal physiological function of cells and play a protective role in sepsis. In the current study, we explore the role of programmed cell necrosis in sepsis and the underlying
mechanisms. The septic rat model is established by Cecal-ligation and perforation (CLP), and the in vitro model is established by LPS in IEC-6 cells. Our results demonstrate that receptor-interacting protein (RIP)1 is significantly upregulated in the ileum of septic rats and LPS-treated IEC-6 cells at both the mRNA and protein levels. Nec-1, an inhibitor of RIP1, reduces the protein levels of RIP1, p-RIP3, and phosphorylated mixed-lineage kinase domain-like (MLKL) (serine 358) and relieves intestinal injury in CLP-induced septic rats with decreased IL-6 and TNF-α levels. The in vitro experiments further reveal that LPS induces the colocalization of RIP1 and RIP3, resulting in the phosphorylation and translocation of MLKL to the plasma membrane in IEC-6 cells. LPS also facilitates ROS production in IEC-6 cells, but this effect is further reversed by Nec-1, si-RIP1 and si-RIP3. Furthermore, LPS-induced necrosis in IEC-6 cells is counteracted by NAC. Thus, we conclude that RIP1/RIP3-dependent programmed cell necrosis participates in intestinal injury in sepsis and may be associated with RIP1/RIP3-mediated ROS.

Keywords: RIP1, RIP3, MLKL, ROS, programmed cell necrosis, sepsis

Introduction

Sepsis is a kind of systemic inflammatory response syndrome (SIRS) caused by infection, the immune response of which is unbalanced and results in multiple organ dysfunction [1]. Its incidence is increasing year by year with rapid progression, and the mortality rate is as high as 30% to 50%. The inflammatory response and oxidative
stress are central to sepsis, which induces cell death and causes irreversible damage to the body [2, 3]. Thus, inhibiting the relevant pathways of the inflammatory response and oxidative stress may help to restore the normal physiological functions of cells and play a protective role against sepsis.

   Programmed cell necrosis is a kind of newly discovered cell death that participates in the occurrence and development of cardiovascular, renal, liver, and nervous system infections, playing an important role in host defense and the inflammatory response [4]. Receptor-interacting protein 1 (RIP1), one of the important members of the silk/threonine kinase family, coupled and phosphorylated homologous protein RIP3, and activated RIP3 further phosphorylated mixed-lineage kinase domain-like protein (MLKL) to promote its membrane translocation, resulting in cell rupture and the occurrence of programmed cell necrosis [5, 6]. Oerlemans et al. [7] reported that necrostatin-1 (Nec-1, RIP1 inhibitor) reduced the inflammatory response and oxidative stress in sepsis. Shashaty et al. [8] found that plasma RIP3 levels were positively correlated with complications and mortality in patients with sepsis, and inhibiting the RIP1-RIP3-MLKL pathway obviously improved the pathophysiological process of sepsis. The above studies suggested that the inhibition of programmed cell necrosis via the RIP1-RIP3-MLKL pathway may be a potential therapeutic target for sepsis, but considering the complexity of the pathogenesis of sepsis, further research is needed.

   In this study, we not only focused on the role of the RIP1-RIP3-MLKL pathway in sepsis both in vivo and in vitro but also explored the underlying mechanisms.
Material and Methods

Animals

Sprague Dawley rats purchased from Vital River (Beijing, China) were used. All experimental procedures in this study were approved by the Animal Committee of the School of Medicine, Xiamen University. The animals were housed in a 22°C room with a relative humidity of 45%-55%, a 12 h day/light period, and food and water *ad libitum*.

The establishment of sepsis model and experimental groups

The sepsis model was established by Cecal-ligation and perforation (CLP) method as previously reported [9], with some changes. Briefly, the rats were anaesthetized with a ventilator before undergoing midline laparotomy to expose the cecum. The cecum was tightly ligated with a 5-0 silk suture at approximately 1/3 of the blind end and perforated twice with a 2.5 mL syringe needle to form four holes. Then, a small volume of feces was extruded through the puncture site after the cecum was squeezed. Finally, the cecum was returned to the peritoneal cavity, and the laparotomy was closed. In the sham group, the rats were submitted to all surgical procedures without ligation and puncture of the cecum. All rats were fed normally after recovery, and they were divided into three groups (n=6): Sham group, Model group, and Model+Nec-1 group. The rats in the Sham group and Model group were intraperitoneally injected with 0.2 mL of normal saline, while the Model+Nec-1 group
was intraperitoneally injected with 0.2 mL of normal saline dissolved in Nec-1 (1 mg/kg; HY-15760; MCE, New Jersey, USA).

**Cell culture and treatment**

Rat IEC-6 cells were obtained from Yaji Biotechnology (Shanghai, China). Dulbecco’s modified Eagle’s medium (DMEM; Procell, Wuhan, China) was used to culture cells in a humid incubator containing 5% CO\(_2\) at 37 °C. For the *in vitro* experiments, a 1×10\(^9\) cell suspension was inoculated into 6-well plates with 4×10\(^5\) cells per well and cultured in an incubator containing 5% CO\(_2\) at 37 °C for 24 h. Then, LPS (10 μg/mL; MCE), Nec-1 (20 μM; MCE), si-RIP1 (20 μM; RiboBio, Guangzhou, China), or si-RIP3 (20 μM; RiboBio) was added, and the cells were cultured for another 12 hours. The sequences of si-RIP1 and si-RIP3 are listed as follows:

si-RIP1: 5′-GGCCAGUAUUGAGAUUGAUTT-3′;

si-RIP3:

5′-CACCGCATCAAGTTGAGGAAGTACGAATACTTCCTCAACTTGATGC-3′.

**Hematoxylin-eosin (HE) staining**

HE kits (G1120; Solarbio, Beijing, China) for HE staining were used according to the manufacturer’s instructions. Briefly, samples for histopathology were removed from the ileum region and placed into neutral buffered formalin for fixation, and 5 μm thick HE-stained sections were prepared following paraffin embedding and histological processing. Finally, images were taken using an Olympus DP70
microscope (Olympus, Tokyo, Japan).

**TUNEL staining**

A TUNEL kit (Beyotime, Shanghai, China) was employed for TUNEL staining according to the manufacturer’s instructions. TUNEL-positive cells were observed under a DM14000 fluorescence microscope (Leica, Wetzlar, Germany). Five fields were randomly selected, and the number of positive cells was calculated for comparison.

**Immunofluorescence**

For the immunofluorescence assay, the ileum section (8 µm) was blocked with goat serum. After blocking, the sections were incubated overnight with an antibody for cleaved caspase 3 at a dilution of 1:800 (#9664; Cell Signaling Technology, Beverly, USA). The next day, after washing, Cy3-conjugated Affinipure Goat Anti-Rabbit IgG (H+L) secondary antibody at a dilution of 1:100 (SA00009-2; Proteintech, Chicago, USA) was added following Hoechst application to each section to stain nuclei. The tissue sections were imaged using the DM14000 fluorescence microscope. Five fields were randomly selected, and the number of positive cells was calculated for comparison.

**Flow cytometry**

IEC-6 cells (1×10^5) were added to 5 µL FITC Annexin V (BD Pharmingen, New
Jersey, USA) and 5 μL PI in the dark for 15 min at room temperature, and cell necrosis was analysed by a flow cytometer (BD FACS Aria, New Jersey, USA).

**Enzyme-linked immunosorbent assay (ELISA)**

IL-6 and TNF-α levels in the serum of rats and cells were measured using a rat IL-6 ELISA kit (E-EL-R0015c, Elabscience, Wuhan, China) and a rat TNF-α ELISA kit (E-EL-R2856c; Elabscience) according to the manufacturer’s instructions.

**Quantitative real-time PCR (qRT-PCR)**

qRT-PCR was performed to measure the mRNA expression of RIP1. The extracted RNA from the ileum and IEC-6 cells was reverse transcribed to complementary DNA using the PrimeScript™ RT Reagent Kit. qRT-PCR was carried out using the SYBR Green PCR Mix Kit according to the manufacturer’s instructions. The reaction conditions were as following: 10 min at 94°C for 1 cycle; then 20 s at 94°C, 20 s at 55°C, and 20s at 72°C for 40 cycle. The results were analysed using the $2^{-\Delta\Delta CT}$ (cycle threshold) method for quantification. The primer sequences of the genes were as follows: RIP1 (F): 5′-AGGTACAGGAGTGTGTTGGTATGGGC-3′, and RIP1 (R): 5′-GGTGGTGCCAAGGAGATGTATG-3′; β-actin (F): 5′-ACCGAGCATGGCTACAGCGTCACC-3′, and β-actin (R): 5′-GTGGCCATCTCTTGGCTCGGAGTCT-3′.

**Western blot analysis**
Total protein was extracted using RIPA lysis buffer, and the concentration of protein was determined using a BCA Protein Assay Kit (Vazyme, Nanjing, China). After that, the protein was separated in a 12% SDS-PAGE gel and transferred to PVDF membranes (Immobilon, Millipore, Bedford, USA). The membranes were then incubated with 5% nonfat milk for 2 h at room temperature followed by incubation with anti-RIP1 (1:1000, 17519-1-AP; Proteintech), anti-RIP3 (1:100, 15828; Cell Signaling Technology), anti-p-RIP3 (1:1000, AF7443; Affinity), and anti-p-MLKL (1:1000, AF7420; Affinity) antibodies overnight at 4°C. The next day, the membranes were incubated with secondary goat anti-rabbit for 1 h at room temperature. Finally, an enhanced chemiluminescence kit (BIO-RADChemiDoc XRS+; Bio-Rad, California, USA) was utilized to visualize the protein bands, and the optical densities of the western blot bands were analysed using Image-Pro Plus 6.0 software. The relative expression of RIP1, p-RIP3, and p-MLKL in the ileum region was normalized to that of β-actin, while the relative expression of p-MLKL in the cell membrane of IEC-6 cells was normalized to that of Na/K ATPase, which was extracted using a Membrane and Cytosol Protein Extraction Kit (P0033; Beyotime).

Confocal microscopy

IEC-6 cells were blocked with goat serum for 30 min after washing with PBS. After blocking, the cells were incubated overnight with anti-RIPK1 (DF2642; Affinity) and anti-RIP3 (sc-374639; SANTA). The next day, after washing, the cells were incubated with anti-rabbit Alexa Fluor 488 (A11070, Thermo Fisher Scientific) for 1
h at room temperature, and a mixture of goat anti-mouse IgG-cy3 (1:200; Proteintech) and anti-rabbit IgG-FITC (1:200; Proteintech) was added to the antibody diluent for 1 h in the dark. Hoechst was then applied to stain nuclei. The cells were imaged and analysed using an SP8 confocal scanning microscope (Leica).

Transmission electron microscopy

The ileum sections of rats were dissected and cut into ultrathin (70-90 nm) sections. After dehydration, ultrathin sections were stained with uranyl acetate (SPI-CHEM) and lead citrate (Sinopharm Group) for observation under an electron microscope. Images were taken using an H-7650 electron microscope (Hitachi, Tokyo, Japan) in a blinded manner.

Coimmunoprecipitation (Co-IP)

The lysates of cells were immunoprecipitated with IP buffer containing IP antibody-coupled agarose beads, and protein-protein complexes were later subjected to Western blotting. For immunoblot analysis, IP products and the input samples were separated by 12% SDS-PAGE, and the target protein was detected by western blot using anti-RIP1 or anti-RIP3, respectively. Here, IgG was used as a negative control.

Dichloro-dihydro-fluorescein diacetate (DCFH-DA) assay

Intracellular ROS level was detected using dichlorodihydrofluorescein diacetate (DCFH-DA, S0033S; Beyotime). Briefly, cells were washed twice with serum-free
culture medium, and 2 mL DCFH-DA (10 μM) was added and incubated in a cell incubator at 37°C for 20 min in the dark. After washing with serum-free cell culture medium and PBS, 1 mL Hoechst 33342 dye was added to the cells and incubated at 37°C for 15 min in the dark. Then, the cells were washed twice with PBS and photographed under an inverted microscope (LEICA DMI 4000B).

Statistical analysis

All experiments were independently repeated at least three times. All the values are presented as the mean ± standard deviation and were analysed by SPSS 22.0 statistical software. Unpaired Student’s t test and one-way ANOVA were used to compare the differences between groups. P < 0.05 was considered statistically significant.

Results

RIP1-dependent programmed cell necrosis participates in intestinal injury in septic rats.

CLP was performed to establish the rat model of sepsis. HE staining revealed that the Sham group exhibited intact epithelial cells, while the Model group exhibited damaged epithelial cells (single arrow), separation between the villous epithelium and lamina propria (red arrow), and vascular dilatation (bold arrow) (Figure 1A). TUNEL staining (Figure 1B) and the immunofluorescence of cleaved caspase-3 (Figure 1C) determined the apoptosis of intestinal epithelial cells in the Model group. Furthermore,
TEM revealed that the structure of intestinal epithelial cells was normal, while the intestinal epithelial cells of the Model group exhibited some necrotic characteristics, including villi detachment, partial mitochondria vacuolation, loss of plasma membrane integrity and intracellular vesicle swelling (Figure 1D). Considering the key role of RIP1 in programmed cell necrosis, we examined the expression level of RIP1 in the ileum of the Sham and Model rats. The qRT-PCR and western blot results revealed that RIP1 was significantly upregulated in the ileum of septic rats at both the mRNA (Figure 1E) and protein levels (Figure 1F). To further confirm the involvement of RIP1 in programmed cell necrosis in the ileum of septic rats, necrostatin-1 (Nec-1), an inhibitor of RIP1, was employed in this study. As expected, pretreatment with Nec-1 obviously reduced the protein levels of RIP1 (Figure 2A). HE staining suggested that Nec-1 significantly improved the damaged epithelial cells, edema, and inflammatory infiltration in the ileum of septic rats (Figure 2B). Consistent with the effect of Nec-1 on histopathology, significantly downregulated levels of IL-6 and TNF-α were also observed in the Model+Nec-1 group (Figure 2C,D). The above results suggested that RIP1-dependent programmed cell necrosis participated in intestinal injury in septic rats.

**RIP1 interacts with RIP3 to induce the phosphorylation and translocation of MLKL to the plasma membrane in IEC-6 cells treated with LPS**

It has been reported that RIP1 activates RIP3 via phosphorylation, and MLKL is further phosphorylated by RIP3 at the threonine 357 and serine 358 residues. Then,
phosphorylated MLKL is translocated to the plasma membrane, resulting in necroptosis [5]. We observed that the protein levels of p-RIP3 and p-MLKL (at the serine 358 residue) were significantly upregulated in the ileum of septic rats, while both were markedly reversed by Nec-1 (Figure 2A). Next, we investigated the effect of RIP1 on RIP3 and MLKL in IEC-6 cells treated with LPS, owing to the elevated necrosis induced by LPS (Figure 3A). Similar to the in vivo results, RIP1 was significantly upregulated in LPS-treated IEC-6 cells at both the mRNA (Figure 3B) and protein levels (Figure 3C). Cellular immunofluorescence further revealed that the colocalization (yellow fluorescence) of red and green fluorescence was significantly increased in the LPS group (Figure 3D), indicating that LPS enhanced the colocalization of RIP1 and RIP3 in IEC-6 cells (Figure 3D). We then investigated the effect of LPS on the phosphorylation and translocation of MLKL to the plasma membrane in IEC-6 cells. As expected, p-MLKL was significantly increased on the plasma membrane of IEC-6 cells treated with LPS (Figure 3E). Additionally, Co-IP further confirmed the direct coupling of RIP1 and RIP3 in IEC-6 cells treated with LPS (Figure 3F). These results suggested that LPS induced the colocalization of RIP1 and RIP3, resulting in the phosphorylation and translocation of MLKL to the plasma membrane in IEC-6 cells.

*RIP1/RIP3-mediated ROS are associated with programmed cell necrosis induced by LPS in IEC-6 cells*
To further confirm the involvement of RIP1/RIP3 in programmed cell necrosis in sepsis, si-RIP1 and si-RIP3 were employed in LPS-treated IEC-6 cells. Consistent with the effect of Nec-1, both si-RIP1 and si-RIP3 significantly reduced LPS-elevated necrosis (Figure 4A), IL-6 (Figure 4B) and TNF-α levels (Figure 4C). We also observed that LPS markedly facilitated ROS production, but this effect was reversed by Nec-1, si-RIP1 and si-RIP3 (Figure 5A). In addition, LPS-induced necrosis was significantly counteracted by N-acetyl-l-cysteine (NAC) (Figure 5B). These results suggested that RIP1/RIP3-dependent programmed cell necrosis may be associated with oxidative stress in sepsis.

Discussion

Sepsis is a hot and difficult topic in the field of critical illness, and its pathogenesis is complicated. Current studies have mostly focused on cell apoptosis induced by the inflammatory response and oxidative stress [10, 11]; however, there are various types of cell death, such as apoptosis, programmed cell necrosis, cell pyroptosis, and cell iron death [12]. The regulation of various types of cell death may help to restore the normal physiological function of cells and play a protective role in sepsis.

Programmed cell necrosis is a kind of cell death that is mainly triggered and activated by the tumor necrosis factor receptor family and toll-like receptor family on the cell surface and is then phosphorylated to activate the important signaling molecules RIP1 and RIP3 to form necrotic complexes. Then, MLKL is activated and
transferred to the plasma membrane, leading to cell energy metabolism disorders and membrane integrity damage, triggering programmed cell necrosis and releasing inflammatory molecules to aggravate inflammation, thus participating in different physiological and pathological processes [13, 14]. At present, there are few studies on programmed cell necrosis and sepsis, but there is evidence that programmed cell necrosis plays an important role in sepsis and may provide a potential target for the treatment of sepsis. Gu et al [15] reported the upregulation of RIP1/RIP3 in liver tissues of LPS-induced septic mice, and activated RIP1 significantly increased RIP1-mediated necroptosis and inflammation. Deng et al [16] reported that ketamine inhibited TNF-α-induced cecal damage by enhancing RIP1 ubiquitination and reducing RIP1-RIP3 and RIP3-MLKL interactions, as well as the formation of necrosomes. However, Mcneal et al [17] reported that Nec-1 treatment in septic mice increased caspase 3 activity. Zhang et al [18] also found that Nec-1 exacerbated liver injury and shortened the survival time of CLP-induced septic rats with increased TUNEL-positive cells and cleaved caspase-3 protein levels. Therefore, the mechanism of programmed cell necrosis in sepsis is still controversial.

Herein, the role of programmed cell necrosis in sepsis was explored in a sepsis rat model (established by CLP) and an in vitro model (established by LPS). The in vivo experiments revealed that Nec-1 markedly reduced the protein levels of RIP1, p-RIP3, and p-MLKL and relieved intestinal injury in CLP-induced septic rats with decreased IL-6 and TNF-α levels. The in vitro experiments further revealed that LPS induced the colocalization of RIP1 and RIP3, resulting in the phosphorylation and
translocation of MLKL to the plasma membrane in IEC-6 cells, which may be responsible for LPS-induced cell necrosis. In addition, LPS markedly facilitated ROS production in IEC-6 cells, but this effect was reversed by Nec-1. It has been reported that TNF induces an increase in intracellular ROS in different cells, depending on RIP1 or RIP3, which is accompanied by the translocation of RIP1 or RIP3, and inhibiting RIP1 or RIP3 significantly reduces ROS levels [19-21]. Furthermore, Shen et al. [22] observed that embryonic fibroblasts of mice with RIP1 knockout resisted hydrogen peroxide-induced cell death. Li et al. [23] reported that RIP1 expression was remarkably upregulated in melanocytes treated with hydrogen peroxide. Not surprisingly, our results revealed that both si-RIP1 and si-RIP3 markedly reversed LPS-induced ROS, and NAC significantly counteracted LPS-induced necrosis in IEC-6 cells. Thus, we concluded that RIP1/RIP3-mediated ROS may be associated with programmed cell necrosis in sepsis. MLKL is also known to be involved in ROS generation [24]. However, unfortunately, we focused on the role of MLKL in LPS-induced necrosis rather than ROS generation in IEC-6 cells.

In summary, the present study demonstrated that RIP1/RIP3-dependent programmed cell necrosis participated in intestinal injury in septic rats, and LPS induced RIP1/RIP3-dependent necrosis via the phosphorylation and translocation of MLKL to the plasma membrane in IEC-6 cells. Mechanically, RIP1/RIP3-mediated ROS may be responsible for programmed cell necrosis. Our findings provide a new idea and direction for the treatment of sepsis.
Acknowledgement

This study was supported by the Natural Science Foundation of Fujian Province Supported Project (No. 2021J011318) and Fujian Provincial Clinical Medical Research Center for First Aid and Rehabilitation in Orthopaedic Trauma, Fujian, China (No. 2020Y2014).

Conflict of Interest

The authors declare that they have no conflict of interest.

References


11. Cicek M, Unsal V, Doganer A, Demir M. Investigation of oxidant/antioxidant and


18. Qin, Zhang, Siwei, Wei, Jiayin, Lu et al. Necrostatin-1 accelerates time to death in a rat model of cecal ligation and puncture and massively increases hepatocyte...


Figure legends
Figure 1. Programmed cell necrosis is induced in the ileum of septic rats  
CLP was performed to establish the rat model of sepsis, and the ileum was collected. (A) HE staining was performed to assess the pathological changes of the ileum of all rats; TUNEL staining (B) and the immunofluorescence of cleaved caspase-3 (C) were performed to examine the apoptosis of intestinal epithelial cells of all rats; (D) TEM was adopted to examine the structure of intestinal epithelial cells; qRT-PCR (E) and western blot (F) were performed to detect RIP1 mRNA and protein levels in the ileum of rats. Data are presented as the mean ± SD. ***P<0.001 vs Sham.

Figure 2. Nec-1 relieves intestinal injury in septic rats  
CLP was performed to establish the rat model of sepsis, and the septic rats were intraperitoneally injected with 0.2 mL normal saline with Nec-1 after the procedures. The rats were sacrificed, and the ileum and serum were collected. (A) Western blotting was performed to assess the protein levels of RIP1, p-RIP3, and p-MLKL in the ileum of rats. (B) HE staining was performed to assess the pathological changes in the ileum of all rats. ELISA was performed to detect IL-6 (C) and TNF-α (D) levels in the serum of rats. Data are presented as the mean ± SD. **P<0.01, ***P<0.001 vs Sham; ###P<0.001 vs Model.

Figure 3. The colocalization of RIP1 and RIP3 induced by LPS facilitates the phosphorylation and translocation of MLKL to the plasma membrane in IEC-6 cells  
IEC-6 cells were treated with 10 μg/mL LPS for 12 h. (A) Flow cytometry was
performed to detect cell necrosis. qRT-PCR (B) and western blot (C) were performed to detect RIP1 mRNA and protein level in IEC-6 cells. (D) IEC-6 cells were subjected to immunofluorescence using anti-RIP1 or anti-RIP3 antibody, the distribution of RIP1 (green) and RIP3 (red fluorescence) was analysed by the immunofluorescence images. (E) Western blotting was performed to examine the protein level of p-MLKL (serine 358) on the plasma membrane of IEC-6 cells. (F) Whole cell extract was immunoprecipitated with an irrelevant rabbit IgG as a negative control or antibody against RIP1, and the immunoprecipitates were then immunoblotted with an antibody against RIP1 or RIP3. Data are presented as the mean ± SD. ***P<0.001 vs Ctrl.

Figure 4. LPS induces RIP1/RIP3-dependent programmed cell necrosis in IEC-6 cells
IEC-6 cells were treated with 10 μg/mL LPS for 12 h, and Nec-1, si-RIP1 and si-RIP3 were employed in IEC-6 cells treated with LPS. (A) Flow cytometry was performed to detect cell necrosis. ELISA was performed to detect IL-6 (B) and TNF-α levels (C). Data are presented as the mean ± SD. ***P<0.001 vs Ctrl; ###P<0.001 vs LPS.

Figure 5. RIP1/RIP3-mediated ROS are responsible for programmed cell necrosis induced by LPS in IEC-6 cells
IEC-6 cells were treated with 10 μg/mL LPS for 12 h, and Nec-1, si-RIP1 and si-RIP3 were employed in IEC-6 cells treated with LPS. (A) DCFH-DA was employed to detect intracellular ROS production. (B) Flow cytometry was performed to detect cell necrosis in IEC-6 cells treated with
N-acetyl-l-cysteine (NAC). Data are presented as the mean ± SD. ***$P<0.001$ vs Ctrl;

##$P<0.01$, ###$P<0.001$ vs LPS.
A

Sham  Model  Model+Nec-1
RIP1
p-RIP3
p-MLKL
β-actin

B

Sham  Model  Model+Nec-1

C

Relative α-SMA level (opt.dpm)

D

Relative TNF-α level (opt.dpm)

160x117mm (600 x 600 DPI)
160x194mm (600 x 600 DPI)
160x104mm (600 x 600 DPI)
Under oxidative stress, the main mode of intestinal epithelial cells in sepsis is apoptosis. Here, we show that RIP1/RIP3-dependent programmed cell necrosis participates in intestinal injury in sepsis, and which may be associated with RIP1/RIP3-mediated ROS. (1) RIP1-dependent programmed cell necrosis is responsible for intestinal injury of septic rats. (2) RIP1 interacts RIP3 to induce the phosphorylation and translocation of MLKL to the plasma membrane in IEC-6 cells treated with LPS. (3) RIP1/RIP3-mediated ROS is responsible for programmed cell necrosis induced by LPS in IEC-6 cells.